Validation of targets and drug candidates in an engineered three-dimensional cardiac tissue model

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High-throughput target discovery confronts the biopharmaceutical industry with a plethora of target candidates. The validation of these candidates in disease-specific animal models often lacks the required throughput. Here, we discuss perspectives and limitations of a novel engineered three-dimensional cardiac tissue, which enables the influence of gene and drug intervention to be monitored on a cellular and molecular level under physiological conditions in sufficient throughput. The model is an extremely helpful filter to prioritize multiple development candidates before moving a project into large animal models with higher predictivity.

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There is increasing evidence of the need for accelerated target validation and drug optimization by the majority of the biopharmaceutical industry. Pharmaceutical companies are anxious to remove bottlenecks in the overall development process and to increase early attrition of drug discovery projects to avoid failure in a later and more expensive stage of drug development.

The 'mining' of the human genome has resulted in a plethora of available genes and potential target candidates, which are presently suffocating any attempts to define new high-quality drug targets in a purely technology driven manner. The drug discovery industry knows of many examples of databases covering a single aspect of a gene, such as differential mRNA expression or single nucleotide polymorphism, which are not able to drive the selection of good target candidates.

The need for validated targets, quality compounds and early attrition

The biological evaluation of targets is the key to their quality. Understanding the biology of a target also means understanding the biology of a disease. That factor not only enables the definition of highest quality targets but also the discovery of quick back-up solutions whenever a target or the corresponding compound should fail in the complex drug discovery process (e.g. assay development, compound screening, hit optimization).

Early access to potential drug candidates and tool drugs is important to accelerate the drug discovery process and could aid the biological characterization of a target. Current miniaturized and cost-effective HTS technologies have enabled the biopharmaceutical industry to feed more targets into the screening process that leads to the discovery of new chemical compounds. This in turn has sparked a new field of research, which was dubbed 'chemical genetics' or 'chemical genomics' [1]. However, the biological profiling of multiple compounds requires robust higher throughput analysis systems.

Predictive biological characterization of targets or compounds requires physiological systems, especially in the quest for novel cardiac therapeutics when the mismatch between the number of target and drug candidates and the suitability of predictive animal models for drug discovery approaches is evident [2–4].

Cardiac models

Transgenic mouse models of congestive heart failure are the predominant tool for mechanistic studies but are considered to be of low predictive value [5]. Aortic banding of the rat heart mimics pressure overload induced heart failure; however, it usually takes 6–12 weeks until heart failure with ventricular dilation occurs [6,7]. Larger animal models, such as

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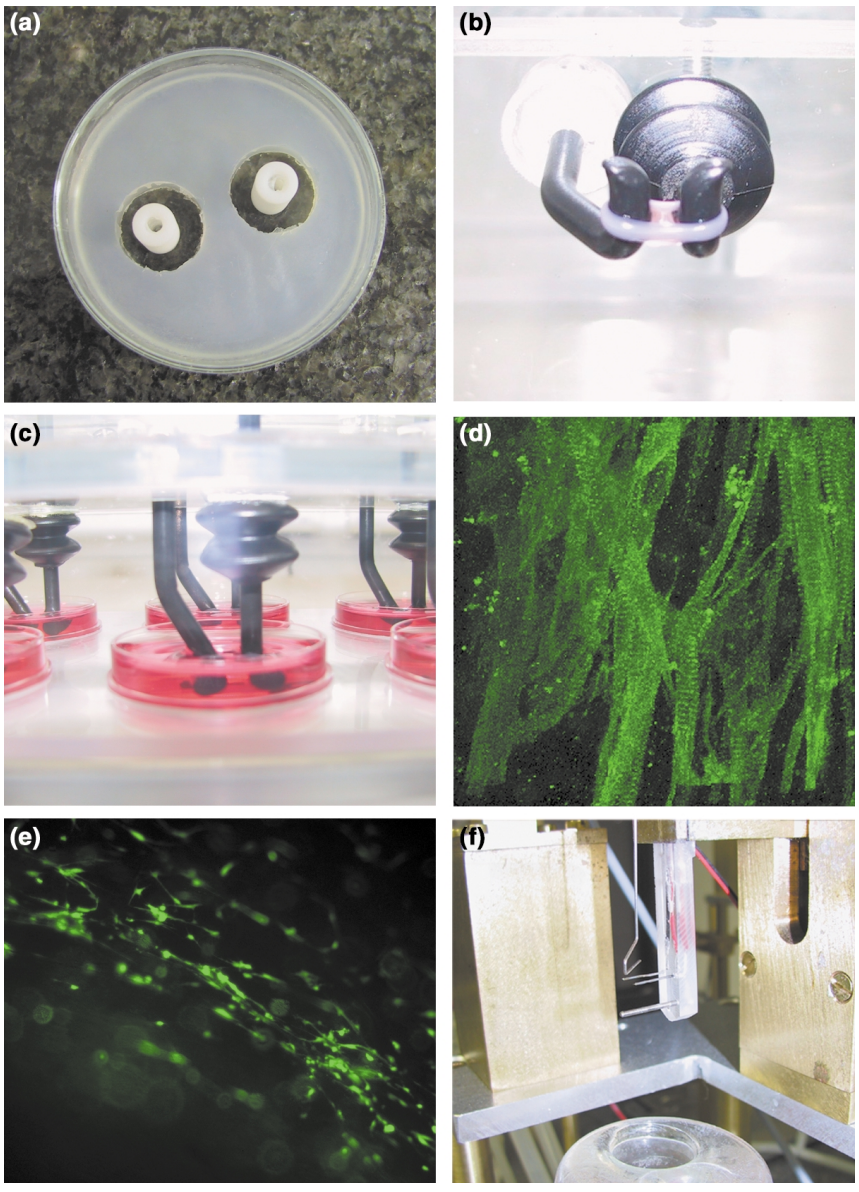

Figure 1. The three-dimensional cardiac tissue model. **(a)** The rings are poured into silicone masks with a centerpiece of 7 mm diameter and incubated for 8 days until they form a tight matrix of ~1mm². **(b)**. Rings mounted on the trainer before the start of training; these are forced to contract for a further 8 days while the tissue matures. **(c)** During this time gene expression can be monitored or drugs applied at appropriate intervals. **(d)** After maturation, rings can be investigated on a cellular or biochemical level. An α-actinin staining of 100μm sections from rings showing the contractile units of heart cells; individual cells can be discerned and the development of a cellular network monitored. **(e)** GFP-tagged target proteins can be expressed in the cells forming the cardiac tissue. The expression of a GFP-tagged protein after 6 days of ring maturation; contraction can be measured in a traditional organ bath system. **(f)** A measurement device designed to hold a ring between the hook and the fixed rod at the bottom of the plastic holder, with pacing electrodes to the left and right. The hook leads to a force transducer designed to measure such low forces which transmits the data to a computer where it is evaluated using specialized software.

tachycardia-induced heart failure in rabbits [8] or microembolization of dogs [9] or pigs [10], clearly have higher predictivity but are restricted in their use to experiments of higher ethical justification and lower throughput, such as preclinical studies for lead compounds.

In vitro approaches, such as the perfused heart according to Langendorff [11] or papillary muscle preparations, are mainly hampered by the limitation of the studies to short-term effects. Janssen and co-workers have succeeded in culturing trabeculae from rabbits *in vitro* for up to 48 h and were able to measure continuous contractions throughout this time without loss of any physiological effects [12]. Although such trabeculae preparations could also be gained from adenovirus infected tissue [13] the system is regarded as a short-term culture method with low throughput.

Cell cultures of neonatal rat cardiomyocytes are an important *in vitro* tool for the molecular analysis of disease parameters, such as cardiac hypertrophy or signal transduction. Although important discoveries, such as the induction of cardiac hypertrophy by α1-adrenergic stimulation, have been made using this model [14], the extrapolation from single cell experiments with neonatal cells to the intact adult heart is limited. One aspect is the clear difference between the immature cardiomyocytes and the adult cells [15,16]. Another aspect is the difficulty in measuring physiological parameters, such as contraction or force in single cells or cellular monolayers because of the random orientation of the single cells. This problem was addressed by Deutsch and co-workers [17], who cultured single cell layers on stretchable microtexture membranes for better attachment and orientation of the cells. However, in these systems the cardiomyocytes are still isolated and not part of a cellular matrix.

Engineered three-dimensional cardiac tissue

To overcome some of the limitations of cell culture, organ preparation and animal experiments, Eschenhagen and

co-workers [18–20] have engineered rat neonatal cardiac myocytes into a contractile three-dimensional (3D) tissue model. This model can be cultured for up to eight weeks and could serve as a bridge between low complexity cell culture and animal experiments.

In the Integrated Target Definition program (MediGene, Martinsried, Germany) for congestive heart failure and other cardiac diseases, this 3D model system is routinely used for the prioritization of target and drug candidates [21]. Tissue rings of ~7 mm diameter are prepared from neonatal rat hearts and a mixture of extracellular matrix proteins using miniaturized casting forms (Fig. 1a). Rings are allowed to form cell–cell contacts and cell–matrix interactions for eight days (Fig. 1b). On day eight, when the rings have established a spontaneous coordinated contraction and rearranged the surrounding matrix to form a tight fabric of ~1 mm^2 in ring cross-sectional area, they are mounted on a trainer (Fig.1c) and forced to exercise in culture medium for 6–8 days. This forced contraction drives the maturation of the cardiomyocytes and the resulting tissue shows molecular characteristics of intact cardiac tissue, such as increased expression of the α-isoform of myosin heavy chain [19,20]. Figure 1d depicts a confocal microscopic picture of a 16-day-old engineered tissue ring showing the directed orientation of the cardiomyocytes, the more mature cellular phenotype and the 3D cellular meshwork. However, a more systematic molecular evaluation of tissue rings before and after maturation and a comparison to adult tissue needs to be completed and is currently under way in our laboratory by Affymetrix Genechip™ analysis and studies of multiple relevant signaling pathways (data not shown).

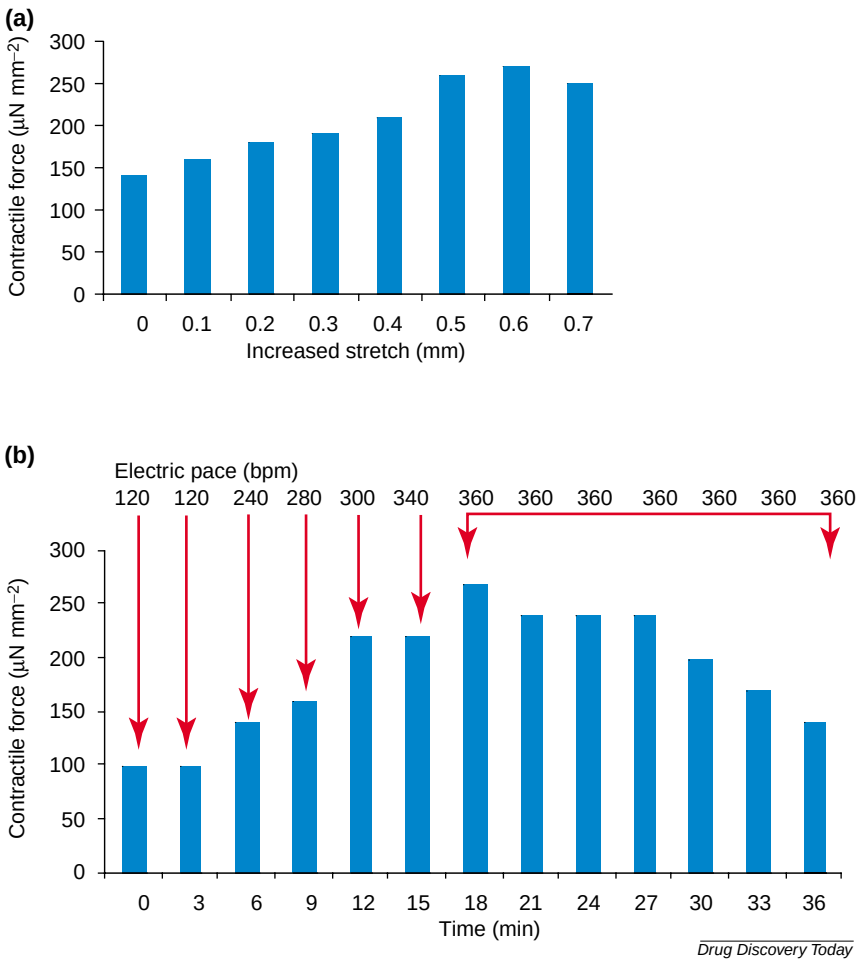

Figure 2. Analysis of physiological parameters of tissoid rings. **(a)** The Frank–Starling effect: the contractile force of rings was measured under increasing preload. The stretch on the rings was increased stepwise by increments of 0.1 mm at constant temperature (37°C) and normal oxygen level. The response is increased contractile force until the ring is overstretched. **(b)** The force–frequency relationship was analyzed by increasing electric pacing from 120 bpm to 360 bpm and measuring contractile force at 37°C. A positive force–frequency can be observed in conditions where the nutrient and oxygen supply is optimal. From 18 min onwards the bpm were kept constant and the force monitored until the rings tired out. From 30 min onwards the oxygen and nutrient supply is limiting and thus the force-frequency is negative from that time onwards.

The engineered heart tissue rings can be transduced with viral constructs for target gene expression (Fig. 1e) or treated with agents such as drugs, cytokines or antisense oligonucleotides. The rings are then transferred into a measurement device and the contractile behavior can be recorded for up to several hours (Fig. 1f).

The contractile force recorded in the tissoid rings varies between 0.2 and 0.8 mN mm^{-2}, which is lower than contractions measured in papillary muscle preparations. The lower contractile power compared with the original heart tissue is possibly a result of differences in cell density, the cellular contacts within the matrix and the connective tissue between the cells. The heart cells arrange themselves within the matrix as a bundle-like network of cells (Fig. 1d), which comprises ~10–20% of the cross-sectional area of the ring, although this is difficult to quantitate. Thus, this system can not achieve the forces achieved in papillary muscle.

Table 1. Drug treatment of cardiac tissue rings

Drug	Concentration	Contractile force (% of control)
Isoproterenol (n = 3)	10 μM	166 ± 19
Amrinone (n = 3)	30 μM	149 ± 4
Prazosin (n = 5)	4 μM	41 ± 9
Metoprolol (n = 3)	20 μM	26 ± 3
Diltiazem (n = 4)	10 μM	37 ± 6
Verapamil (n = 6)	5 μM	28 ± 26
Nifedipine (n = 6)	10 μM	17 ± 6
Adenosine (n = 3)	95 μM	63 ± 19
Lidocaine (n = 4)	100 μM	45 ± 5

However, the important interpretations of this system are the relative changes in, for example, contractile force caused by overexpressed target proteins or drug substances compared with the respective controls and thus the lower overall force compared with other muscle preparations is irrelevant.

Similarity to physiological tissue

To further evaluate how close the tissoid system is to the physiology of muscle or whole heart preparations, we analyzed parameters such as the Frank–Starling behavior of the rings [20] – which states that the more force is exerted on a muscle the more forcefully it contracts – and the force–frequency relationship. The force measurement device enables the adjustment of the degree of stretch or 'preload' of the ring tissue using a micrometer wheel. We were able to monitor a tissoid behavior according to the law of Frank–Starling. Figure 2a shows that it was possible to increase the contractile force by nearly 100% by increasing the preload from a basal value – reflecting ~15% overstretch of the tissue – to a maximum preload – representing ~30% overstretch. Exceeding the preload to higher values results in a loss of contractility, possibly because of disruption of the tissoid.

The response of the tissoid to electrical stimulation is illustrated in Fig. 2b. The endogenous rhythm of the tissoids varies between 60 and 160 bpm depending on the preparation. Under experimental conditions the rings are paced with a constant stimulus of 120 bpm. To investigate the force–frequency relationship, tissue was stimulated from the basal value of 120 bpm up to 360 bpm, which resulted in a 170% increase in force generation. Prolonged stimulation at a higher pace led to the reduction of force. Our observations are consistent with the reports of Zimmermann *et al.* [20] who also showed a positive length–force relationship and a positive force–frequency behavior at physiological parameters, but negative force frequency in stressful situations, such as low oxygen or loss of energy stores or after prolonged high frequency stimulation (Fig. 2b). This resembles responses of intact rat heart preparations in organ bath systems [22,23]. Following measurement, rings can be kept in culture for repeated studies or processed for the analysis of histology, gene expression or signal transduction.

Pharmacological effects on a cardiac tissue model

The pharmacological response of cardiac tissue to calcium, isoprenaline, carbachol and pertussis toxin was described by Zimmermann and colleagues, demonstrating the basic calcium activation of cardiac cells and the functional presence of β-adrenergic receptors, muscarinic receptors and inhibitory G-proteins (Gi/Go) [20].

To further characterize the pharmacological properties of the cardiac tissue rings, the effects of additional pharmacological agents on cardiomyocytes under constant pacing of 120 beats per minute were analyzed (Table 1; Navé *et al.*, unpublished data).

β-Adrenergic signaling

Isoproterenol, a non-selective β-adrenergic agonist that lowers peripheral vascular resistance but exerts positive inotropic (changes in force) and chronotropic (changes in frquency) effects on the heart, increased the total force of the rings to 166 ± 19% of the value before treatment with the drug. Interestingly, the maximal force was increased, whereas the basic tension of the rings (the diastolic force) was decreased (Fig. 3a; Becker *et al.*, unpublished data).

Amrinone, a cAMP phosphodiesterase type III inhibitor, interferes with a more downstream component of the β-adrenergic signaling cascade. The compound increases the cellular levels of cAMP and exerts a positive inotropic response, which was monitored in the tissue model by an increased force to 149 ± 4% (Fig. 3b; Navé *et al.*, unpublished data).

The effects of prazosin, a selective α1-adrenergic receptor antagonist, were measured in a reduction of the overall force to 41 ± 4%, and metoprolol, a β_1-selective adrenergic receptor antagonist, also showed a strong negative inotropic action (26 ± 3% of the untreated value).

Calcium channels

Commonly used inhibitors of the slow Ca^{2+} channels (L-type) are diltiazem, nifedipine and verapamil. Verapamil is known to block the L-type Ca^{2+} channel and to increase the refractory period. This leads to a depressed rate of the sinus

node and a slowing of the AV-node conduction. In addition, the fast Na^+ current is inhibited, resulting in a direct strong negative inotropic, chronotropic and dromotropic (changes in contraction time) effect [24,25]. Diltiazem also depresses the rate of the sinus node and slows AV conduction. In the *in vivo* situation, there is a biphasic response to diltiazem. Initially the vasodilatory action is dominant. The results are reduced peripheral vascular resistance and increased coronary perfusion, leading to increased cardiac output. Subsequently, the direct negative inotropic effect becomes dominant [26].

Nifedipine usually shows a more pronounced vasodilatory action resulting not only in increased coronary blood flow but also in a marked decrease in arterial blood pressure [26]. This invokes a compensatory sympathic response, which leads to tachycardia and positive inotropism. However, the direct *in vitro* effect is a negative inotropic one (Fig. 3c). For all three compounds the expected effects on the cardiac tissue were demonstrated, with a reduction of the force to 37 ± 6% for diltiazem, 28 ± 26% for verapamil and 17 ± 6% for nifedipine, respectively.

Na^+ channels

Lidocaine, widely used as an intravenous antiarrhythmic drug, directly interferes with the fast Na^+ current, leading to a lower resting membrane potential, which results in a higher threshold for excitability and in a reduced force–frequency relationship. A negative inotropic effect was observed in the cardiac tissue model with a force reduced to 45 ± 5%, which is in accordance with reports describing reduced ventricular rate after lidocaine administration in patients with ventricular arrhythmias [27].

K^+ channels

Adenosine activates an acetylcholine sensitive K^+ current in the sinus and AV nodes and leads to a shortening of the action potential duration. Additionally, binding of A3-receptor agonists reduces ryanodine binding and decreases the rate constant of calcium release from the sarcoplasmic reticulum [28]. In agreement with these data we monitored a reduction of force to 63 ± 19% in our 3D model perfused with adenosine.

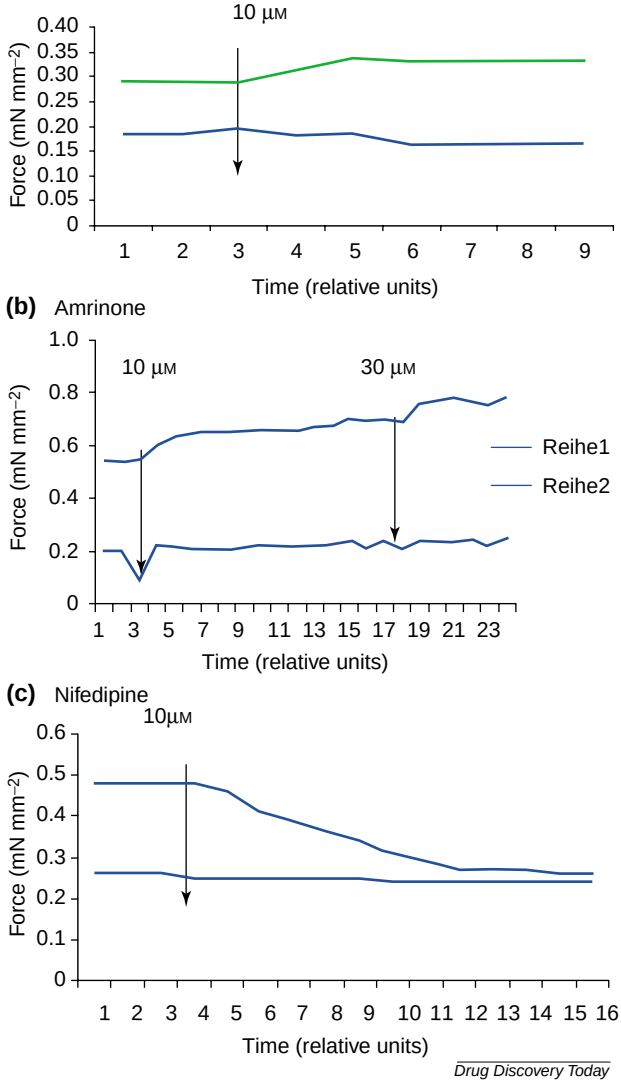

Figure 3. Drug influence on force generation in 3D model. Panels **(a–c)** show the change in contractile force after administration of various compounds at the concentrations indicated in each graph. Force readings were taken at the time points indicated and recorded as mN. Indicated in these graphs are the basal tension (lower line) and the maximal contraction (upper line) of the ring. Therefore, the difference between the two lines gives the absolute contractile force for each ring. The arrows indicate the time at which the drug was administered. Each graph depicts one representative experiment. The averages for each experiment are given in Table 1.

Target validation in a cardiac tissue model

In analogy to drug treatment, the model is suitable for the validation of target candidates. Manipulating the gene expression level of an interesting target candidate within the tissue model can be achieved by efficient transduction with viral constructs carrying the target gene, a dominant mutant or an antisense cDNA. Zimmermann and colleagues showed highly efficient transduction of the reconstituted cardiac tissue using adenovirus-mediated gene transfer [20]. Our own studies have shown that the system enables us to study long-term effects of gene expression for up to 6 or 8 weeks (Fig. 1e; Becker *et al.*, unpublished data). Parameters that can be studied are force generation, as described previously, histology, cell morphology, hypertrophy, signal transduction and gene expression.

Limitations and perspectives of engineered tissue models

Tissue engineering is a technique that combines the use of biological and engineering expertise to generate a limitless amount of tissue from small samples. In the past decade, the field of tissue engineering has made tremendous progress. The lack of autologous grafts and the rejection of xenografts have triggered the investigation of *in vitro* grown tissue and 'organoids' [29]. The first medical applications, such as healing of large and chronic wounds using engineered skin grafts or the replacement of cartilage, each with comparatively simple architectures, have already reached the patient [30]. In aortic valve disease mechanical heart valves will be increasingly replaced by bioengineered valves because of better performance [31,32]. Tissue engineered artificial urothelium could evolve into a superior option for urological surgery and bladder wall replacement [33,34]. Recently, nerve grafts of engineered Schwann cells have been produced from neuroma and have been used successfully in animal models [35,36].

In contrast to the use of engineered tissue in replacement therapy, few reports describe the use of *in vitro* reconstituted tissues and organoids in mechanistic studies. Chakir and co-workers engineered a bronchial mucosa, which showed pseudostratified ciliated epithelium with the presence of mucin-secreting cells and was used for studies of inflammatory cells in asthma [37]. Rat liver with albumin-producing hepatocytes was reconstituted *in vitro* by Takezawa *et al.* and the biochemistry of the liver cell was studied in this system [29]. Damour and colleagues proposed the use of engineered skin models for pharmacological and toxicological analysis of cosmetic products [38].

The engineered cardiac tissue model described in this article addresses some of the needs of modern drug discovery: the monitoring of basic cellular and physiological parameters in a standardized higher throughput system. The extrapolation of the generated data to the intact heart, or even to the whole organism, is limited. The work of Eschenhagen and co-workers [18–21] and our own data show that some physiological and pharmacological effects are nicely reflected. However, whereas the direct effects of verapamil or nifedipine, for example, on the heart tissue can be monitored, the strong vasodilatory effects of the compounds can not be addressed in a non-vascularized system. The generated forces are clearly lower than in comparable muscle preparations as discussed above and many cellular and molecular characteristics of the system still need to be elucidated. One of the most urgent questions, which is currently being addressed, is the degree of maturation of the trained tissoid in comparison with adult tissue.

Nevertheless, this long-term model enables us to address many mechanistic questions and to prioritize genes and compounds before entering into predictive large animal models, which for ethical reasons are not amenable for these routine experiments.

The future

The near future will see a dramatic improvement in engineered tissue models by miniaturization and combination with other tissue models, for example, vascularized cardiac models or innervated skin models. Another perspective is the introduction of *in vitro* differentiated mammalian stem cells into the tissue models, which will increase the throughput and will further decrease the variability of the current models, which mostly depend on primary cells [21].

Engineered tissue models will capture an important role in the modern drug discovery process where biology meets chemistry in an early phase of development.

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